INFECTIOUS MULTIPLE DRUG RESISTANCE IN THE ENTEROBACTERIACEAE

ANNUAL REPORT

By

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The applicability and limitations of examining clinical specimins with a DNA hybridization technique for genes encoding enterotoxins was examined using 24 heat-labile and heat-stable (LT^+ST^+), 17 heat-labile (LT^+ST^-), and 22 heat-stable (LT^-ST^+) enterotoxigenic Escherichia coli isolated in Thailand. Enterotoxigenic E. coli were identified with the Y-1 adrenal and suckling mouse assays. All were homologous and thus identifiable with radiolabled fragments of DNA encoding for heat-labile toxin (LT) or heat-stable toxins of

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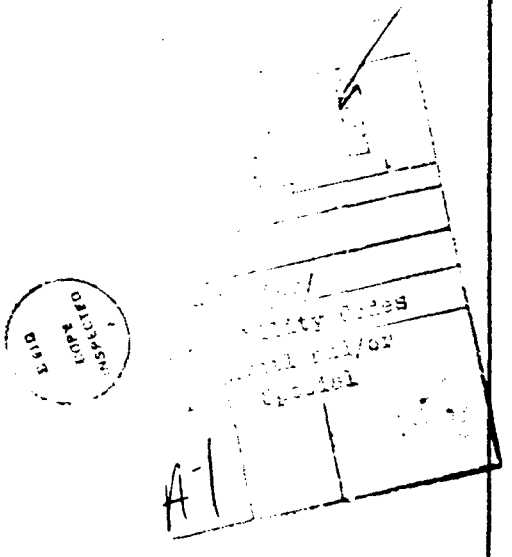
porcine (ST-P) and human origin (ST-H). $LT^{-}ST^{+}$ strains from rural Thailand were homologous with only ST-H and not ST-P, while strains isolated in Bangkok were homologous with either ST-H, ST-P, or both. The hybridization technique detected DNA homologous with the three probes in bacterial growth of all stools from patients with diarrhea from whom enterotoxigenic E. coli were isolated and in enterotoxigenic E. coli-inoculated water containing other species of bacteria. The DNA hybridization assay is a useful technique for characterizing enterotoxigenic E. coli and identifying environmental sources of these enteric pathogens.



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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

Preface

This research contract, which has been continuously supported by the U.S. Army Medical Research and Development Command since July 1972, was completed on 1 July 1981, with the departure of the Principal Investigator from the University of Washington to Stanford University. An Annual Progress Report was submitted to USAMRDC in January 1981, covering the period of 1 January 1980 through 1 January 1981. This annual report covers the period of 1 January 1981 through 1 July 1981. It concentrates upon studies performed in cooperation with the U.S. Army Medical Laboratory in Bangkok, Thailand. In addition, we have appended a complete list of all research completed and published under contract support, as well as reprints of articles not previously submitted to the USAMRDC.

The Principal Investigator wishes to express his thanks to the U.S. Army for their continuing confidence in the research efforts of his laboratory.

Abstract

The applicability and limitations of examining clinical specimens with a DNA hybridization technique for genes encoding enterotoxins was examined using 24 heat-labile and heat-stable (LT^+ST^+), 17 heat-labile (LT^+ST^-), and 22 heat-stable (LT^-ST^+) enterotoxigenic Escherichia coli isolated in Thailand. Enterotoxigenic E. coli were identified with the Y-1 adrenal and suckling mouse assays. All were homologous and thus identifiable with radiolabelled fragments of DNA encoding for heat-labile toxin (LT) or heat-stable toxins of porcine (ST-P) and human origin (ST-H). LT^-ST^+ strains from rural Thailand were homologous with only ST-H and not ST-P while strains isolated in Bangkok were homologous with either ST-H, ST-P, or both. The hybridization technique detected DNA homologous with the three probes in bacterial growth of all stools from patients with diarrhea from whom enterotoxigenic E. coli were isolated and in enterotoxigenic E. coli-inoculated water containing other species of bacteria. The DNA hybridization assay is a useful technique for characterizing enterotoxigenic E. coli and identifying environmental sources of these enteric pathogens.

Key words: Enterotoxin, E. coli, Plasmid, Nucleic acid, Hybridization

Introduction

Over the past decade there has been a growing appreciation that bacterial plasmids may directly contribute to pathogenicity.[1,2] The best known examples of plasmid-mediated factors of pathogenicity are the enterotoxins and colonization factors of certain E. coli serotypes.

Enterotoxigenic E. coli are important causes of diarrhea in infants, children and adults in developing countries and also traveller's to these countries. The capacity of these strains to produce enterotoxins and cause disease is largely plasmid-mediated. Plasmids, called Ent,[3] encode for two general classes of enterotoxins, a heat stable enterotixin (LT) which is structurally and functionally similar to cholera toxin as well as several types of heat stable enterotoxin (ST) which are non-immunogenic, small polypeptides (about 47 amino acids) which act by stimulating the production of increased levels of cyclic GMP in small bowel cells leading to fluid secretion into the bowel lumen.[4,5]

Plasmids may carry genes for only ST, only LT or both ST and LT.[2,3,6] While Ent plasmids are generally transmissible by cell-to-cell contact to recipient cells in the laboratory, naturally occurring isolates carrying Ent plasmids are usually restricted to a small handful of E. coli serotypes.[7] Thus the Ent plasmids do not appear to be widely distributed in nature. In part, this may be a misleading finding since microorganisms carrying only an Ent plasmid are often avirulent. Rather, in order for E. coli to be fully pathogenic, the presence of yet another plasmid species (or plasmid gene), the colonization or Kad plasmids, are required.[1,2,3]

The Kad plasmids encode for proteinaceous cellular appendages which appear as bacterial pili on the cell surface. These pili adhere to certain mammalian cells, especially small bowel epithelial cells. It is the combination of both the Kad pili biosynthesis and toxin production that is necessary for enteropathogenicity in E. coli.[3] To be sure, other factors are involved. One cannot simply transfer an Ent plasmid and Kad plasmid to any E. coli to produce a pathogenic strain.[3] Nevertheless, we view plasmid-mediated toxins and colonization factors as a general microbial strategy to produce disease.

Despite the global importance of enterotoxigenic E. coli as a major cause of diarrheal disease, the epidemiology of infection is not well understood. One impediment to the study of E. coli diarrhea has been the difficulty in differentiating enterotoxigenic strains from normal flora. Current methods involve the detection of enterotoxin production by biological and immunological assays. E. coli LT is detected by tissue culture[8,9] and immunological assays[10] while ST is assayed by fluid accumulation in ligated rabbit[11] or pig[12] intestional loops or in infant mice.[13] All of these techniques require the preparation of cultural supernatants of individual strains or pools of strains to be assayed for toxin production. The cost and inconvenience associated with these methods make them unsuitable for large-scale epidemiological studies.

In our last contract report, we devised a DNA hybridization method which can detect the presence of genes encoding the ST and LT enterotoxins. At that time we noted that (a) not all ST-producing strains could be detected with our

available gene probes, but that (b) we had been able to isolate by recombinant DNA methods a new sub-class of ST gene. In this annual report, which covers the period 1 January, 1981 - 30 June, 1981, we present our findings of the applicability cell limitations of the DNA hybridization method for identifying enterotoxigenic E. coli. This work was performed at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, in collaboratin with Dr. Peter Echeverria, as well as workers from Mahidol University in Bangkok. The work has been submitted and accepted for publication in the "Journal of Infectious Diseases".

Results

1. Identification of Enterotoxigenic E. coli by DNA Hybridization: Report of a Field Study in Bangkok.

a. Plan of the Study

Last year we reported in the "Journal of Infectious Diseases"[14] that it is possible to detect enterotoxigenic strains of E. coli using radioactive enterotoxin gene-specific DNA fragments and DNA-DNA hybridization. Briefly, E. coli strains (or fecal material from patients) are inoculated onto the surface of a nitrocellulose filter which has been overlayed onto a suitable solid growth medium. After growth of the bacteria, the cells are lysed with NaOH and the DNA is fixed on the filter. The filter is then incubated with radioactive ST or LT DNA (probe DNA) under conditions permitting DNA-DNA duplex formation with any homologous DNA sequences on the filter. The filter is then washed and placed against X-ray film. Enterotoxigenic strains (or fecal material containing enterotoxigenic microorganism) are detected by exposure of the film over areas of the filter where DNA-DNA duplexes have formed, indicating the presence of DNA sequences homologous to the toxin gene.

During these initial studies we could detect all LT-producing bacteria. However, using a DNA probe from an ST gene isolated from an E. coli of porcine origin, ST-P, we could detect only 11% of LT^+ST^+ strains and 71% of LT^-ST^+ E. coli. Subsequently, we isolated a second ST gene from an E. coli of human origin. This sequence has been named ST-H and, as we reported in last year's Annual Report, differs significantly in DNA sequence from ST-P.

Mr. Steve Moseley, a graduate student in my laboratory, travelled to the Armed Forces Research Institute of Medical Sciences Bangkok, Thailand, in April 1981. His goal was to examine the applicability and limitations of examining clinical specimens by DNA hybridization techniques and to ascertain the utility of the ST-H and ST-P probes together for the identification of ST-producing cells. Initially enterotoxigenic E. coli strains were collected at random from children and adults with diarrhea at Children's Hospital, and Bamrasnaradura Infectious Disease Hospital, Bangkok, Soongnern Hospital, Soongnern, and Peace Corps volunteers at four different locations in a rural Thailand. Strains from animals were isolated in a rural village in Soongnern district. Ten lactose-positive colonies, selected from primary isolation plates of each clinical specimen were tested for LT and ST in the Y-1 adrenal cell assay and the suckling mouse assay respectively. These latter assays served as a standard for comparison of the DNA probe method.

In a separate study, stools or rectal swabs were collected from children less than two years of age. These patients all had watery diarrhea of less than 72 hours duration and were seen at the Children's Hospital in Bangkok

during a one-week period. The stools or rectal swabs were cultured simultaneously on MacConkey agar and spotted in triplicate on nitrocellulose filters. After overnight incubation at 37 C, ten lactose-positive colonies were selected from the MacConkey plate and tested within two weeks of isolation for LT by the 41 adrenal cell assay and ST in the suckling mouse assay. The nitrocellulose filters were hybridized with the LT, ST-P and ST-H probes as described in last year's Annual Report and in a subsequent publication.

b. Homology of ETEC with DNA Probes

Twenty-four LT^+ST^+, 17 LT^+ST^- and 22 LT^-ST^+ E. coli were examined for homology with the LT, ST-P and ST-H DNA probes. The sources of the isolates are given in Table 1. All 41 LT^+ST^+ and LT^+-ST^- E. coli were homologous with the LT probe. Hence, as in our Dacca study of 1980, we were able to accurately identify all strains which gave positive adrenal cell tests for LT. An interesting feature not previously observed came out of these Thai studies, however. Five E. coli strains considered to be positive in the adrenal cell assay by the Armed Forces Research Intitute personnel were not identified by the LT probe. Once this disparity was observed, these E. coli were examined in more detail. Sterile culture filtrates of these E. coli did, in fact, cause rounding of Y-1 adrenal cells in tissue culture. This activity was not inhibited by V. cholerae anti-toxin nor with E. coli anti-LT sera. Thus these strains do not produce LT, nor do they stimulate adenyl- cyclase activity typical of E. coli LT. The activity that is observed may be a cytotoxin or some other topic moiety. These data affirm, therefore, the utility and specificity of the LT probe to identify toxigenic LT-producing microorganisms.

All of the LT^+ST^+ and LT^-ST^+ E. coli were detected with either the ST-P and/or the ST-H DNA probes. Among LT^+ST^+ isolates, 11 were homologous with ST-P, 11 were homologous with ST-H and two strains were homologous with both. These data are in contrast to that obtained in the 1980 Dacca study when only 23% to 50% of LT^+ST^+ strains were identified. The difference, of course, is the availability of the ST-H probe in this study. By the same token, we could only identify 72% of LT^-ST^+ strains in the 1980 Dacca experiments. In this study, we identified every strain that was positive in the suckling mouse model. Of the 22 LT^-ST^+ E. coli examined, 13 were detected with ST-H, six with ST-P and three with both probes.

Genes homologous with either the ST-P or ST-H probes were distributed unequally among isolates from different sources. LT^-ST^+ E. coli homologous with ST-H but not ST-P were only found in individuals with diarrhea in rural Thailand. The difference between the proportion of LT^-ST^+ E. coli homologous with the ST-P probe from rural verses urban Thailand (9/9 vs 4/12) was significant ($p = 0.03$ [Fisher's exact test]; see Table 2). All 7 of the LT^-ST^+ strains isolated from children with diarrhea in urban or rural Thailand were homologous with only the ST-H probe.

c. Detection of ETEC infection in patients with diarrhea.

One hundred-and-ten stools were examined simultaneously with the DNA hybridization assay and by testing 10 lactose-positive colonies for LT and ST by the adrenal cell assay and suckling mouse assay respectively. The same eight children were found to be infected with ETEC by both methods. Two children were infected with LT^+ST^+, 5 with LT^+ST^- and one with LT^-ST^+. The number of colonies detected by the adrenal cell and suckling mouse assay varied from 1/10 to 10/10.

d. Effect of other microorganisms on detection of ETEC

While it was clear that DNA probes could detect ETEC when present in stools at a low proportion of the total lac+ cells, it seemed useful to get a rather more precise idea of the sensitivity of the method. Different proportions of nonenterotoxigenic E. coli or A. hydrophila were mixed with LT^+ST^+ E. coli B2C cells to determine to what extent the DNA of other bacteria might interfere with the detection of genes encoding for LT and ST. The DNA hybridization assay was positive when 10^9 A. hydrophila or non-enterotoxigenic E. coli were mixed with 10^5 LT^+ST^+ E. coli B2C and spotted on a nitrocellulose filter which was placed on MacConkey agar and incubatd at 37 C for 2+ hours. Mixtures containing only A. hydrophila or non-enterotoxigenic E. coli were uniformly negative.

To further determine the sensitivity of the DNA probe assay for the detection of ETEC in water, ten-fold dilutions of LT^+ST^+ E. coli B2C were made in klong (canal) water. One ml of klong water contained 2.1×10^5 Proteus sp., 1.5×10^5 Enterobacter sp., 1.3×10^5 A. hydrophila, 6×10^4 non-toxigenic E. coli; 6×10^4 klebsiella pneumoniae and 3.2×10^4 Pseudomonas sp. The DNA probe assay was uniformly positive when ETEC were diluted in klong water to contain 10 ETEC/ml. When 10 ml was passed through a 0.45) millipore filter which was placed on the surface of a MacConkey agar plate and incubated at 37 C overnight.

Discussion

All LT^+ST^+, ST^+ST^- and LT^-ST^+ E. coli characterized as enterotoxigenic by standard assays by the Armed Forces Research Institute in Bangkok were detected with our available DNA probes for enterotoxin genes. As in our previous work, all LT-producing E. coli were homologous to a single LT DNA probe suggesting that the structural genes encoding for LT are closely related among strains from a variety of sources. In our previous work with a single ST-P probe, we could detect 21% of LT^+ST^+ strains and 71% of LT^-ST^+ bacteria. In this study, we included an additional ST probe, ST-H, and all strains as positive in the suckling mouse were identified by hybridization. The observation that some strains of ETEC were detected by both the ST-P and ST-H probes indicate that some strains possess two genes encoding ST production. Preliminary data suggest that in most (if not all) of these strains the two genes reside in different plasmids. The use of the ST gene probes should therefore be of value in the study of the molecular epidemiology and dissemination of Ent plasmids.

By examining ETEC from different sources with the ST-P and ST-H probes, it was recognized that this technique may be useful in defining the epidemiology of ETEC. LT^-ST^+ strains in rural Thailand were homologous with only the ST-H and not the ST-P probe while strains in Bangkok were homologous with either probe or both. Furthermore, all LT^-ST^+ E. coli isolated from children were homologous with ST-H. In Bangladesh, 70% of LT^-ST^+ E. coli were homologous with the ST-P probe as compared with 27% of similar strains isolated from Thailand ($p<.001$; chi-square). Although the number of strains examined were not sufficient to draw firm conclusions, examining more strains in different populations and environments should help clarify the epidemiology of these enteric pathogens.

Since the vast majority of water and other environmental sources frequently contain many species of bacteria, we were concerned that DNA from

other microorganisms might interfere with the detection of DNA coding for enterotoxin. Spotting stools directly on nitrocellulose filters fixing the bacterial DNA contributed by the fecal flora and examining filters with our ^{32}P-labelled probes was certainly as sensitive as the current method of testing 10 individual colonies for enterotoxin production. Similarly we could easily detect ETEC in water containing 10 ETEC/ml. These experiments indicate there is little concern that DNA from other species of bacteria would interfere with the detection of genes coding for toxin even when the total mass of DNA from non-toxigenic bacteria on the filters far exceeds the amount of DNA coding for enterotoxin.

The DNA hybridization assay should serve as a useful tool in defining the epidemiology of ETEC infections. A major advantage of this assay is that it differentiates between the two heterologous genes encoding ST. One of the more difficult bacteriological problems in defining the epidemiology of ETEC in the field is in the separation of *E. coli* from the bacterial biomass present in most environmental sources. The DNA hybridization assay allows specimens containing numerous other species of bacteria to be screened directly for the genes encoding enterotoxins.

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Table 1

Source of Enterotoxigenic Escherichia coli isolated from individuals with diarrhea

No. of ETEC		Source
24 LT^+ST^+	10*	Children, Children's Hospital, Bangkok
	6	Children, Soongnern Hospital, Soongnern
	4	Adults, Bamrasnaradura Hospital, Nonthaburi
	3	Peace Corps volunteers, rural Thailand
	1	Adult, Soongnern Hospital, Soongnern
17 LT^+ST^-	7	Children, Children's Hospital, Bangkok
	3	Children, Soongnern Hospital, Soongnern
	3	Adults, Soongnern Hospital, Soongnern
	2	Peace Corps volunteers, rural Thailand
	1	Buffalo, Soongnern
	1	Pig, Soongnern
2 LT^-ST^+	10	Adults, Bamrasnaradura Hospital, Nonthaburi
	5	Children, Soongnern Hospital, Soongnern
	3	Adults, Soongnern Hospital, Soongnern
	2	Children, Children's Hospital, Bangkok
	1	Peace Corps volunteer, rural Thailand
	1	Pig, Soongnern

* Number of individuals from whom ETEC were isolated (one ETEC/individual)

Table 2

Homology of ST-P and ST-H with LT^+ST^+ and LT^-ST^+ *E. coli* isolated from patients with diarrhea in urban and rural Thailand

Source	No. of ETEC isolated	No. of colonies homologous with		
		ST-P	ST-H	ST-P + ST-H
Urban†	14 LT^+ST^+	5	8	1
	12 LT^-ST^+	5	4	3
Rural‡	10 LT^+ST^+	6	3	1
	9 LT^-ST^+	0	9	0
TOTAL	24 LT^+ST^+	11	11	2
	21 LT^-ST^+	5	13	3

LT^+ = produce heat-labile toxin

ST^+ = produce heat-stable toxin

† Urban refers to metropolitan Bangkok

‡ Rural refers to specimens collected from Thais in Soongnern and American Peace Corps volunteers at U'thong, Pranburi, Inburi, and Sri Prachan

One LT^-ST^+ *E. coli* isolated from a pig in rural Thailand was only homologous with ST-P.

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